Specification

Anti NC1 Monoclonal Antibody

Technical field

The present invention relates to a detecting method and a detecting reagent for diagnosis of nephritis using anti NC1 monoclonal antibody. Furthermore, therapeutic implements and medicines are embraced therein.

Background art

Main conventional parameters to detect or diagnose nephritis using the urine sample have included protein, albumin, type IV collagen (three chains-domain) and β 2M etc. Further, conventional definite diagnostic method for nephritis has depended on such method to stain renal slice samples obtained by renal biopsy and to observe existing deposit of immunoglobulin(hereafter referred to as Ig) or formation of crescents. For example, diagnostic criteria of IgA nephropathy includes only one method to perform renal biopsy as its definite diagnosis. More specifically, it can be diagnosed in such manner that "granular deposit of IgA mainly in diffused mesangial region of kidney" can be detected by immunofluorescence or enzyme immunoassay (p.1071,[Laboratory Examination : 2001~ 2002] published by BUNKO-DO).

Disclosure of invention

Problems to be solved by the invention

These techniques, however, include the following problems:

The former parameters mentioned above are excellent ones for conventional diagnosis of nephritis, but it still remains that these parameters cannot be applied to elucidate nephritis essentially because they cannot indicate what antigen may affect to deposit of Igs in glomeruli where progressing various nephritis.

The latter definite diagnosis has needed high diagnostic technique by well experienced pathologists. In addition, at the time when the deposit of Igs is detected, the stage of the nephritis might have already progressed in long period, sometime for several decades and its renal function might have remarkably decreased. Therefore,

such diagnostic method has been aspired to diagnose nephritis more simply and more precisely as diagnosing at very early stage when pathological findings such as deposit of Ig and/or formation of glomerular crescent cannot be obtained.

Means for settlement of the problems

The present invention can diminish such weak points as mentioned above and dedicates detection methods and detecting reagents for diagnosing nephritis in its early stage and further dedicates a sero-cleaning method.

The present inventors, heretofore, have suggested that the common antibody among various nephritis is NC1 domain of type IV collagen. In fact, the present inventors have detected anti NC1 antibody in high frequency in the serum or urine through immune response in various nephritis as well as anti glomerular basement membrane(GBM) antibody nephritis. On the other hand, it has been reported that the antigen of anti GBM antibody nephritis exists in NC1 domain and exists normally at inner the domain but the antigen, however, will be expressed from outer the domain at the crisis and will be bound with the autoantibody. There is, however, no antibody prepared artificially can be bound with the antigen only at the crisis but the autoantibody can. Furthermore, existence of a common antibody in various nephritis cannot have been detected by immunohistofluorescent stain method. Thereupon, the present inventors prepared an anti NC1 monoclonal antibody by sensitizing the antigen NC1 isolated and purified from bovine renal glomeruli with the mouse and accomplished "Anti NC1 monoclonal antibody" and " Labeled anti NC1 monoclonal antibody" which are applicable to Western blot method or immunofluorescent stain method, and further accomplished "NC1 detecting ELISA kit with sandwich technique" equipped with "Anti NC1 monoclonal antibody".

The procedures are as follows:

1. [Isolation and purification of antigen] Isolate type IV collagen NC1 domain (hereinafter referred as NC1) from the raw material of bovine renal glomeruli and purify it by column chromatography (J. Biol. Chem., 263, 10481-8).

2. [Preparation and selection of anti NC1 monoclonal antibody] Prepare monoclonal antibody using the mouse by the usual method (Monoclonal Antibody Experimental

Manual., published by KODAN-Sha, 1987).

For screening of the cell fusion, select a lot of positive pores with high antibody titer by ELISA technique and further select the antibody responding to NC1 monomer and/or NC1 dimmer by Western blot technique. Then, perform immunofluorescent stain and firstly select the antibody responding to monkey anti glomerular basement membrane (GBM) antibody nephritis and secondarily select the antibody not responding to normal monkey kidney. Thus can obtain the anti NC1 monoclonal antibody applicable to not only ELISA method, Western blot method but also immunofluorescent stain method.

Of course, such anti NC1 monoclonal antibody is preferable as applicable to only one method or two or more methods among ELISA, Western blot, immunofluorescent stain or other methods. Such anti NC1 monoclonal antibody, however, is most preferable as applicable to any method. In immunofluorescent stain method, such antibody responding to normal kidney as polyclonal antibody prepared from the rabbit etc. is acceptable and useful to detect existence of the tissues. However, it cannot be used to discriminate between nephritis and normal kidney.

A crab-eating macaque nephritis model prepared hereupon consists with a manner of 1mg of initial dose and 3mg of booster dose of NC1 by dorsal injection different from the previous reports using plantar injection. The merits of dorsal injection includes no difficulties in walking and less infectious in dipedal walking in the animal. More specifically, in the dorsal injection, it is preferable to inject the larger booster dose compared to the initial dose rather than 4mg of single dose or smaller or equivalent booster dose compared to the initial dose. Further specifically, more than 1.5-fold booster dose is more preferable and 3-fold is most preferable compared to the initial dose.

This theory is usefully applicable to preparation of type II collagen arthritis etc. of sensitized animal models or to various vaccinations. For example, in the case of vaccination of hepatitis type B, the conventional booster dose, sometime, may not increase its antibody titer. In such case, more than 1.5-fold booster dose compared to the conventional single dose or 1/2 initial dose and 3-fold booster dose compared to the

conventional initial dose are more preferable.

"Anti NC1 monoclonal antibody" of the present invention stains renal glomerular basement membrane(GBM) of the pathological sections obtained from monkey GBM antibody nephritis or human IgA nephropathy using indirect immunofluorescent stain method. Furthermore, this antibody stains similarly other pathological sections obtained from other animal species such as the rat and mouse etc. or other various human nephritis rather than IgA nephropathy. "NC1 assay ELISA kit" of the present invention is useful to early detection of primary nephritis and secondary nephritis such as diabetic nephritis etc. More specifically, in " Anti GBM antibody nephritis", the anti NC1 monoclonal antibody reacts significantly with NC1 which is abundant in GBM at injury crisis. Therefore, it can obtain particularly good hypersensitivity and contribute to sensitive detection of NC1 in urine as well as in serum.

Furthermore, anti NC1 antibody in serum or urine of HIGA mouse IgA nephropathy model can be detected by ELISA method with "Anti NC1 monoclonal antibody" as the positive standard. In any case of glomerular nephritis such as other nephritis, diabetes, hypertension model etc., assay or detection of anti NC1 antibody may be a significant indicator of the disease progression. In the other cases of nephritis occurred in infection or other disease models, assay or detection of anti NC1 antibody may also be a good indicator of the disease progression.

The present inventors established the following operative means for "NC1 assay ELISA kit" in order to detect nephritis in early stage. More specifically, the detecting method and the assay reagents in order to detect NC1 using the urine sample obtained from anti GBM antibody nephritis patient as well as using the serum sample will be exemplified and detailed below. However, the present invention should not be construed as being limited thereto.

1. NC1 detecting method and assay reagents in serum and/or urine.

NC1 can be detected using the following reagents: 1) Anti NC1 antibody(derived from rabbit) coated plate, 2) Enzyme(HRP) labeled anti NC1 monoclonal antibody, 3) Coloring substrate(TMB), 4) Reaction stopper solution(sulfuric

acid).

Hereupon, it is preferable to prepare non-labeled "2)" and add "Enzyme(HRP) labeled anti mouse IgG antibody" as "2)-2". Further, it is preferable to exchange their antibody parts between "1)" and "2)" or to change the both into monoclonal antibody.

Hereunto, the positive standard is preferably obtained from the human patient but more preferably obtained from the monkey of experimental model invented by the present inventors. Because the more stable standard can be obtained from the monkey which is more carefully bred and well-controlled. More specifically, it is preferable to make the human patient sample to a primary standard and the sample derived from the monkey to a secondary standard actually equipped in the assay kit.

Immune reaction assay methods include not only enzyme immunoassay method which is representatively used but also AB method, RIA method, immunoluminescence method, precipitation method, agglutination method etc. An enzyme labeled antibody in enzyme immunoassay includes the both antibodies without distinction as to a polyclonal or monoclonal antibody. Furthermore, the antibody can be preferably equipped into a radio-labeled compound(RIA method), lumino-labeled compound(immunofluorescence method) or non-labeled compound(precipitation method, agglutination method).

The reaction mode includes not only a sandwich method but also a competitive method etc. The sandwich method is, however, specifically more preferable. For a composition of assay reagents, an anti NC1 antibody coated plate can preferably be made of glass or magnetic substance, or a method without solid phase method not using plate is also preferably accepted.

When anti NC1 antibody(hereinafter referred to as the antibody) is coated on a plate, such coating substance can preferably be indirectly coated on a plate as adipic acid, biotin, or combined substance of them.

An antigen can preferably be prepared from not only bioextracts or recombinants but also constructive peptides (including specified fractions or synthesized materials). Then the antibody can preferably be prepared from these antigens.

The animal species of antigens using for the assay reagents include preferably not only human but also monkey, bovine, swine, chicken, sheep, goat, rabbit, rat etc, but not limited to them. Further, the antigen can preferably include mixed antigens from multi-animal species.

The antigen derived organ includes preferably the kidney, but not limited to it.

2. Anti NC1 antibody remover and/or NC1 remover.

NC1 in sera can only be removed by dialysis of the blood through an affinity column prepared with anti NC1 antibody and subsequently anti NC1 antibody in sera can only be removed by dialysis of the blood through an affinity column prepared with NC1. After these treatments, the blood removed both antibody and antigen will be recycled into the internal circulation of the body. Application of this principle to the crab-eating macaque nephritis model {sensitized by [K35 NC1](provided by Collagen Research Center)} resulted showing less than halves in concentrations of both antigen and antibody in urine compared to their concentrations before treatment. On the other hand, in conventional dialysis, concentrations of both the antigen and antibody in the patient serum may not show such differences between before and after treatments as mentioned above. Of course, any remover of NC1 and/or anti NC1 antibody in serum may preferably be included, but not limited to the above mentioned removers. Further, it is preferable to replace NC1 or anti NC1 antibody with α3-chain group, α4-chain group and/or their antibodies. It is also preferable to replace them with α3-chain antigen part, α4-chain antigen part inducing anti GBM antibody nephritis and/or their antibodies.

Antibodies used in the remover include preferably either polyclonal or monoclonal antibody and monoclonal antibody is more preferable because it can be processed with semi-permanently constant activity in its property.

The removers of the present invention are specifically useful for such nephritis needed emergency as anti GBM antibody nephritis etc.

Effect of the invention

The present invention is useful for early detection or definite diagnosis of nephritis and for recovery of nephritis patient or cancer patient.

Example 1

[Isolation and purification of antigen] Isolate NC1 domain of type IV collagen (hereafter referred to as NC1) from bovine renal glomeruli as the raw material and purify it using column chromatography (J. Biol. Chem., 263, 10481-8).

[Preparation and selection of antibody] Prepare antibodies using the mouse (Monoclonal Antibody Experimental Manual. KODAN-Sha. 1987). For screening of cell fusion, select a lot of positive pores which possess high antibody titer from the cultured supernatant using ELISA method. After cellular proliferation of them in the mouse abdominal cavity, collect the ascitic liquid and select the antibody which reacts with both NC1 monomer and NC1 dimmer (Fig.1). Then, perform immunofluorescent stain using crab-eating macaque normal kidney and nephritis model kidney (anti GBM antibody nephritis) and select the antibody which reacts with the glomeruli of the crab-eating macaque anti GBM antibody nephritis but not with the crab-eating macaque normal kidney(Table 1, Fig. 2).

(The crab-eating macaque nephritis model of the present invention prepared hereupon, is different one in its administration site from the reported administration site previously. In this model of the present invention, the administration consists of 1 mg NC1 of the primary dorsal injection and 3 mg NC1 of the booster dorsal injection. Dorsal injection may not produce any difficulty in walking or in bipedal walking of the monkey and cause less infectious compared to a plantar injection.)

[Experiment on NC1 injection in crab-eating macaque (female, 3 y.o.)] 2 animals in each group.

1) Administration site and method; The same doses of both NC1 and FCA were injected intracutaneously on the dorsal site of the animal.

2) Assay of anti NC1 antibody titer in the urine (diluted into 50-fold)(Assays at before administration and 4 weeks after initial administration)

	Changes of titer mean values of 2 animals
· Single administration (4 mg)	0.018 ⇒ 0.087
· Twice administrations (3 mg initial, 1 mg booster after 3 weeks)	0.029 ⇒ 0.256

· Twice administrations (1 mg initial, 3 mg booster after 3 weeks)

0.006 ⇒ 1.037

3) Assay method; Urinal specimens were added onto a 96 wells micro-plate applied with NC1(5 μ g/ml) derived bovine and incubated for 2 hours at room temperature. After washing, HRP labeled anti human IgG antibody was added and incubated for 1 hour at room temperature. After washing, a luminary substrate solution was initially added and after 10 minutes, a reaction stopper solution was secondarily added. Then, the absorbance at 450 nm of wave length (A450 nm) was immediately measured.

Furthermore, an anti human type IV collagen (antigen derived from placenta, pepsin treated) polyclonal antibody(derived from rabbit) and an anti NC1 polyclonal antibody(derived from rabbit) were prepared and their stainability was compared between in a monkey normal kidney and in a monkey nephritis model kidney using an indirect immunofluorescence method. Consequently, both the above kidneys were stained (Fig.3-1, Fig.3-2). On the other hand, the anti NC1 monoclonal antibody of the present invention is more stainable in nephritis kidney rather than in normal kidney.

Therefore, the anti NC1 monoclonal antibody of the present invention dedicates an useful staining reagent in order to distinguish nephritis.

In the fact, the anti NC1 monoclonal antibody selected in such manner based on the present invention can stain the kidney of human glomerulonephritis. For instance, the antibody can stain GBM and uriniferous tubule of the frozen kidney section from IgA nephropathy.

The antibody of the present invention can stain kidneys in various human nephritis not only in IgA nephropathy but also in renal GBM, more specifically, glomeruli, uriniferous tubule, Bowman's capsule etc. in a minimal-change type primary nephrosis or a diabetic nephropathy. However, it does not stain kidneys which include those of a recovered minimal-change type nephrosis or normal kidney (Fig.4). Furthermore, in order to confirm the specificity of this antibody, when its immunological response was examined by Western blot method using type IV collagen (derived from human placenta, pepsin treated) and NC1(derived from bovine renal

glomeruli, collagenase treated) as antigens, the antibody responded to NC1 but did not respond to type IV collagen(Fig. 5). The anti NC1 monoclonal antibody of the present invention can be applicable either to ELISA method, Western blot method or to immnofluorescent stain method.

Example 2

[Anti NC1 antibody remover and/or NC1 remover]

NC1 in sera can only be removed by dialysis of the blood through an affinity column prepared with anti NC1 antibody and subsequently anti NC1 antibody in sera can only be removed by dialysis of the blood through an affinity column prepared with NC1. After these treatments, the blood removed both such antibody and antigen will be recycled into the internal circulation of the body. In accordance with this principle, the blood equivalent to 4 ml of serum was collected from a nephritis model of crab-eating macaque (female, approx. 3 y.o.) and dialyzed through two kinds of affinity columns described above and then, recycled into the internal blood circulation of the animal and this procedure was subsequently repeated 3 times. When the both antibody titers in urine before and after treatment were assayed, the antibody titer after treatment decreased into less than half compared to that before treatment.

Example 3

[Type IV collagen assay kit prepared with anti type IV collagen antibody made from type IV collagen antigen derived from kidney]

It is well known existing of chains from α1-chain to α6-chain in type IV collagen. Constitution of α-chains may differ in the kinds of sourcing organ. Type IV collagen derived from placenta possesses mainly α1- and α2-chains. On the contrary, type IV collagen of kidney origin possesses α3- and α4-chains abundantly compared to the placenta origin. In order to obtain type IV collagen derived from kidney of the present invention, extract the collagen from bovine renal GBM using usual pepsin-degradation method. Then, remove NC1 fine particles, which may be contaminated in this process, using anti NC1 affinity column prepared separately. Consequently, pure type IV collagen derived from kidney can be obtained through this process. At the same time, the antibody with specifically high titer can be obtained by

making the type IV collagen to an antigen.

Measurement of antibody titer;

Coat the antigen (1)Bovine renal glomeruli pepsin soluble type IV collagen, 2)human placental pepsin soluble type IV collagen) onto 96 wells plate and add 100 μl of specimen. After incubation for 2 hours at room temperature, add HRP labeled antibody (each antibody in which anti mouse antibody if the specimen is derived from mouse, anti rabbit antibody in derived from rabbit or anti goat antibody in derived from goat) and incubate them for one hour at room temperature. Add TMB solution and incubate them for 10 minutes at room temperature and then stop the reaction by 1N sulfuric acid stopper solution. Then, measure immediately the absorbance at 450nm of wave length.

Specimens and assay results;

- Specimen/Anti human placental type IV collagen monoclonal antibody (Immune animal/mouse, 3 kinds; 1A, 1B, 1E) ;
    1) All minus(background deleted, the same bellow)
    2) 1A/1.826, 1B/2.188, 1E/2.222
- Specimen/Anti human placental type IV collagen polyclonal antibody (Immune animal/rabbit, YOKO203) ;
    1) 2.391
    2) 2.231
- Specimen/Marketed anti human placental type IV monoclonal antibody (Immune animal/mouse, F59)
    1) 0.047
    2) 2.135
- Specimen/Marketed anti human placental type IV polyclonal antibody (Immune animal/goat, GOAT) ;
    1) 0.450
    2) 2.037
- Only antibody YOKO responded to both bovine renal GBM pepsin soluble type IV collage and human placental pepsin soluble type IV collagen but other antibodies

responded to only one of these type IV collagens.

Conclusion;

For the measurement of renal type IV collagen, the reagent prepared with the antibody against renal type IV collagen antigen (ELISA kit etc.) is preferable.

Further, the renal type IV collagen antigen is preferable to use in assaying anti type IV collagen antibody for evaluation of renal function.

Example 4

[Assay of anti NC1 antibody in IgA nephropathy model HIGA mouse]

3 HIGA mice (female, 4 week aged) were purchased and bred for measurement. Test specimens were obtained from one sampling for blood specimen and several samplings per day for urine totalized specimen. The serum sample was diluted into 200-fold and the urine sample into 4-fold for measurement. ELISA method was used for assay.

- Both IgA antibody and IgG antibody were detected in the sera of all animals aged 6 weeks or more.
- IgA antibody was detected in the urine of the animals aged 15 weeks and IgG antibody was detected in the urine of the animals aged 18 weeks.

Brief description of the drawings

Fig.1. Selection of antibody by Western blot method: Lane 17: Control; Poly.: Anti NC1 polyclonal antibody.

Fig.2. Comparison of staining with anti NC1 monoclonal antibody between crab-eating macaque normal kidney and its nephritis model kidney (Indirect immunohistofluorescent stain).

Fig.3-1. Comparison of staining with anti human type IV collagen (derived placenta) polyclonal antibody (derived rabbit) between crab-eating macaque normal kidney and its nephritis model kidney (Indirect immunohistofluorescent stain).

Fig.3-2. Comparison of staining with anti NC1 polyclonal antibody(derived rabbit) between crab-eating macaque normal kidney and its nephritis model kidney Indirect immunohistofluorescent stain).

Fig.4. Staining of kidneys of various human nephritis with anti NC1

monoclonal antibody (Indirect immunohistofluorescent stain).

Fig.5. Western blot method using both antigens of type IV collagen (derived human placenta, pepsin treated) and NC1(derived bovine renal glomeruli, collagenase treated):

anti NC1 mono 12D: anti NC1 monoclonal antibody;

anti NC1 poly: anti NC1 polyclonal antibody;

anti type IV: anti human type IV collagen polyclonal antibody;

control: no addition of the primary antibody

Table 1. Staining procedure

Table 1.

Materials;

Frozen renal tissues of crab-eating macaque anti GBM antibody nephritis model and normal one. (Embed into OCT compound, freeze rapidly using dry ice-acetone or liquid nitrogen, preserve at −80℃).

Antibody;

Primary antibody: Anti NC1 monoclonal antibody(derived mouse);

Secondary antibody: FITC labeled anti mouse antibody(derived rabbit)

(DAKO Inc., Code No. F0232, Lot. 045).

Procedure;

1) Prepare frozen slice pieces using Cryostatt
2) After drying, fix them for 5 minutes with acetone
3) Wash with phosphoric acid buffer solution(PBS, pH 7.4)
4) Incubate for 2 hours at room temperature with the primary antibody (diluted into 500-fold solution)
5) Wash with PBS
6) Incubate for 1 hour at room temperature with the secondary antibody (diluted into 50-fold solution)
7) Wash with PBS
8) Mount with glycerol